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Involvement of nitric oxide in chondrocyte cell death in chondro-osteophyte formation

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Summary

Objective: To examine the nitric oxide (NO) production relevant to chondrocyte cell death in order to elucidate the mechanism of chondro-osteophyte formation in osteoarthrotic joints.

Design: Human chondro-osteophytes were obtained during total hip arthroplasty. Expression of inducible nitric oxide synthase (iNOS) mRNA was determined by *in-situ* hybridization. Localization of iNOS and nitrotyrosine at protein level were examined by immunohistochemistry. Cell death of chondrocytes were confirmed by both TUNEL method and transmission electron microscopy.

Results: The various populations of proliferative and hypertrophic chondrocytes expressed iNOS mRNA and iNOS as well as nitrotyrosine protein. Approximately 30% of hypertrophic chondrocytes forming chondro-osteophyte showed positive reaction to TUNEL staining. Electron microscopy confirmed both disintegrated and apoptotic chondrocytes in these zones. In the deep hypertrophic zone calcification was seen around each of the matrix vesicles and some masses of cell debris.

Conclusion: Chondro-osteophyte formation involves NO production by chondrocytes. The expression and localization of iNOS and nitrotyrosine in chondro-osteophytes suggest the significant role of NO in chondrocyte hypertrophy and apoptosis. © 2001 OsteoArthritis Research Society International

Key words: Nitric oxide, Chondrocyte, Apoptosis, Chondro-osteophyte, Osteoarthrosis.

Introduction

Articular margins contain periosteal osteoprogenitor cells and in osteoarthrotic (OA) joints these cells react to mechanical, physical and chemical stress. Chondro-osteophyte (also known as osteochondrophyte or osteophyte) is a unique neoplastic tissue commonly seen at the peripheral area of OA joints[1]. The role of chondro-osteophytes is a limited regeneration or repair of the affected joint to avoid further damage and widening of the weight bearing surface or stabilizing the joint[2]. Chondro-osteophytes are composed of fibrocytes, osteoblasts, prechondrocytes, and chondrocytes that express type I, IIA, and IIB procollagen mRNA, respectively[3]. The histological appearance resembles normal enchondral ossification at the epiphyseal plate in a growing skeleton or healing fracture callus. Although analysis of experimentally-induced chondro-osteophytes by intraarticular injection of papain[4] or partial menisectomy[5] suggests a role for mechanical, chemical and hormonal transducers in the process of chondro-osteophyte formation, the exact mechanism leading to the formation of this tissue has not been fully determined.

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Apoptosis is an important cellular event and is involved in the maintenance, remodeling, or turnover of mature articular cartilage. A variety of morphological and biochemical studies have identified apoptotic chondrocytes in both growth plates and diseased articular cartilage[6–11]. Recent studies have shown that inducible nitric oxide synthase (iNOS), synthesized by OA chondrocytes in response to inflammatory cytokines such as interleukin (IL)-1 or tumor necrosis factor (TNF)-α, catalyses the formation of nitric oxide (NO)[9,12–15]. NO can cause chondrocyte DNA strand breaks, modify lipids and proteins and induce chondrocyte apoptosis.

Our study was designed to elucidate the mechanism of chondro-osteophyte formation in OA joints. For this purpose, we examined NO production by human chondrocytes obtained from chondro-osteophytes in order to correlate its role in chondrocyte hypertrophy and apoptosis.

Materials and methods

TISSUE PREPARATION

The present study was performed on a series of 21 specimens from 15 individuals (aged 46–82 years, average, 62.7 years) who were treated by total joint arthroplasty for OA lesions of the hip. Thin slices (5 mm) of chondro-osteophytes were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.3) for 24 h at room temperature. Three of the 21 blocks were embedded in glycol methacrylate semimer

without decalcification. The other 18 blocks were decalcified by 0.3 M EDTA (pH 7.5) for 7–10 days. Each block was divided into two blocks, and half of them were embedded in paraffin. After light microscopic examination, smaller blocks (1×1 mm) containing chondro-osteophytes were cut from the remaining half of the blocks, and embedded in hydrophilic resin (LR-White).

HISTOLOGY

Paraffin sections (4.5 μm in thickness) from all 18 blocks were stained with safranin O-fast green to detect the proteoglycan sites which indicate synthesis by chondrocytes. Undecalcified tissue sections from three individuals were stained by Von Kossa method to identify the calcium-containing calcified matrix. They were then observed by light microscopy.

IN SITU DETECTION OF APOPTOSIS

In situ apoptosis was detected in 15 specimens from 15 cases using the modified method described previously[16]. Briefly, sections were digested with 0.1% trypsin containing 0.1% $CaCl_2$ for 30 min at room temperature before testicular hyaluronidase digestion, followed by treatment with 0.3% H_2O_2 in methanol. DNA was end-labeled with biotin-conjugated deoxyuridine triphosphate (dUTP) using terminal deoxynucleotidyle transferase (TDT). The avidin-biotin-peroxidase complex (ABC) method (Nichirei Corporation, Tokyo, Japan) was carried out for 30 min, treated with 3,3′-diamino-benzidine (DAB) in H_2O_2 (0.01%) for 5 min to develop reaction products, and counterstained with methyl green.

PREPARATION OF RNA PROBES

Digoxigenin-11-UTP-labeled single RNA probes were prepared using DIG RNA Labeling Kit (Boehringer Mannheim) according to the instructions provided by the supplier. To generate human iNOS probe, mRNA from synovial tissue sample obtained from a patient with rheumatoid arthritis was extracted using TRIzol, a 0.416 kb fragment of human iNOS cDNA (GenBank database, accession No. U05810) was obtained by reverse transcription followed by polymerase chain reaction (RT-PCR) and was subcloned into Bluescript I pKS.

IN SITU HYBRIDIZATION FOR iNOS mRNA

Paraffin sections (4 μm thick) were pre-treated by proteinase K (1 mg/ml and incubated for 15 min at 37°C). DIG-labeled probe was diluted by hybridization buffer and incubated for 16 h at 50°C in a humidity chamber saturated with 50% formamide. After immunoreaction with anti-DIG antibody diluted ×1000, coloring solution containing 337.5 μg/ml of NBT and 165 μg/ml of BCIP in DIG buffer 3 was mounted on the sections and incubated at 37°C until the signal–noise ratio was maximum (usually 2–3 h), and counterstained by methyl green.

IMMUNOHISTOCHEMISTRY

Paraffin sections from 15 cases were pre-incubated in testicular hyaluronidase (1 mg/ml for 30 min at room temperature) for digestion of glycosaminoglycans in the cartilage matrix. Sections were immersed in 3% H_2O_2 in methanol to prevent endogenous peroxidase reactions, and treated with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 30 min to eliminate non-specific binding. They were then incubated with rabbit polyclonal anti-human iNOS antibodies (10 μg/ml) (Chemicon International, Incorporated, Temecula, CA), or rabbit polyclonal anti-human nitrotyrosine antibodies (10 μg/ml) (Upstate Biotechnology Incorporated, Lake Placid, NY) diluted with PBS containing 0.1% NaN_3 and 0.1% bovine serum albumin (British Biocell International Co.) overnight at 4°C. After thorough rinsing, sections were incubated with biotinylated goat anti-mouse IgG for 1 h at room temperature. Sections were then treated by the ABC method for 30 min, DAB in H_2O_2 (0.01%) for 5 min, and counterstained with methyl green. Negative control sections were treated by non-immune serum as a substitute for the primary antibody, or in the same fashion but excluding the primary antibody. They were then examined under a light microscope.

SEMI-QUANTITATIVE ANALYSIS

Twelve specimens from 12 cases which contained distinct zonal differentiation of chondrocytes were selected for semi-quantitative analysis of TUNEL, iNOS and nitrotyrosine positive cell ratio. The remaining three specimens from three individuals showed relatively late stage chondro-osteophytes with bony areas and no clear zonal differentiation, and were excluded from the analysis. The total number of chondrocytes, and the number of chondrocytes staining positive for TUNEL method or the specific antigens for iNOS and nitrotyrosine were counted within the proliferative and hypertrophic zones under light microscope (×40; Olympus, Japan). The percentage of staining positive cells were expressed: (−) no cells staining, (+) 0–25% of cells positive, (++) 26–50% of cells positive, (+++) >50% of cells positive.

TRANSMISSION ELECTRON MICROSCOPY

Ultrathin sections were first stained by toluidine blue and examined by light microscopy. Adjacent sections were then contrasted with aquenous uranyle acetate and lead citrate, and examined by a transmission electron microscope (TEM) (type 7100, Hitachi, Tokyo).

Results

Histologically, chondro-osteophytes consisted of a central core of bone capped by fibrocartilage on the surface and hyaline cartilage, which could be divided into proliferative, hypertrophic and calcified zones. The hypervascular surface fibrocartilage consisted of undifferentiated mesenchymal cells and collagen fibrils. Cells forming the chondro-osteophyte showed chondrogenic differentiation, from mesenchymal cells to pre-chondrocytes, proliferative chondrocytes, and hypertrophic chondrocytes in the cartilaginous area. At the lower hypertrophic zone, chondrocytes swell further, appear to degenerate or die, and are invaded by capillaries from the bone marrow [Fig. 1 (A,C)]. Calcium-containing calcified matrix stained by Von Kossa method were distributed over the hypertrophic zone, with dense staining at the cartilage-bone junction [Fig. 1(b)].

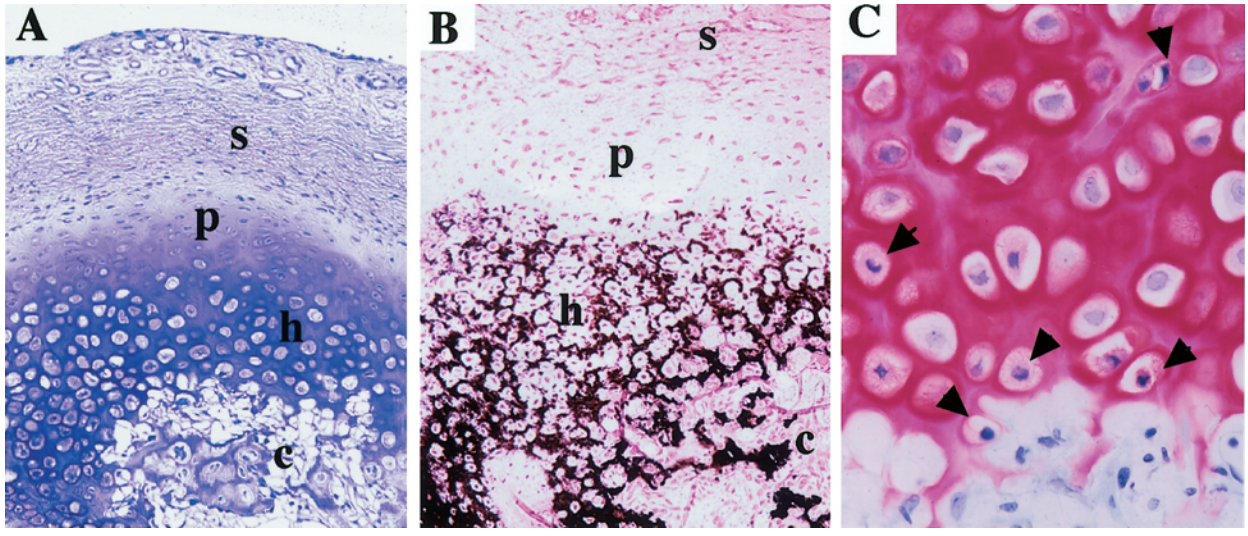

Fig. 1. Histology of the chondro-osteophyte. (A) Semi-thin section embedded in LR-White stained by toluidine blue. Chondrocytes follow chondrogenic differentiation of mesenchymal cells to proliferative and hypertrophic chondrocytes. s: surface fibrocartilage, p: proliferative zone, h: hypertrophic zone, c: calcified zone. Original magnification: ×33. (B) Calcium-containing calcified matrix stained by Von Kossa method. Note the distribution of calcified area over the hypertrophic zone of chondro-osteophyte. Original magnification: ×33. (C) A high-power micrograph of the deep hypertrophic zone stained by safranin O showing proteoglycan-rich matrix of this zone. Arrow: apoptotic chondrocytes with nuclear condensation. Original magnification: ×132.

TUNEL-positive cells ranged from 12.8 to 31.3% (mean, 22.8±5.7%) in the proliferative and from 23.5 to 37.5% (mean, 30.3±4.5%) in the hypertrophic zones [Fig. 2(a,b)]. No positive reaction was seen in the superficial zone. *In situ* hybridization showed a significant expression of iNOS mRNA on proliferating and hypertrophic chondrocytes [Fig. 2(c,d)]. Immunohistochemically, iNOS positive chondrocytes were distributed in the similar area [Fig. 2(e,f)]. Nitrotyrosine was also localized in proliferative and hypertrophic chondrocytes, indicating the presence of cytotoxic peroxynitrite [Fig. 2(g,h)]. Results of semi-quantitative analysis for TUNEL stain, immunostain for iNOS and nitrotyrposine on each of the 12 paraffin embedded sections are summarized in Table I. The nitrotyrosine and iNOS positive ratios varied among the 12 specimens, but the TUNEL positive ratio was relatively fixed.

EM examination showed typical apoptotic changes mainly in cells of the hypertrophic zone. The nucleus appeared small and pyknotic, and the cytoplasm contained electron-dense materials [Fig. 3(a,b)]. Cells located in the deepest layers of the hypertrophic zone were fragmented or showed degenerative changes with massive cell expansion [Fig. 3(c,d)]. In the calcified zone, early calcification was noted around each matrix vesicle derived from viable chondrocytes, or around aggregates of apoptotic bodies [Fig. 3(e,f)].

Discussion

The underlying pathogenic mechanism of chondro-osteophyte formation is unknown at present but several etiologic mechanisms have been proposed. Moskowitz and Goldberg[5] postulated that the mechanical force acting on the site of soft tissue attachments at the joint margin plays a primary role in osteophyte formation. Marshall and Olsson[17] indicated that chondro-osteophytes are formed adjacent to the joint cartilage through the process of chondroid metamorphosis of fibrous tissue, followed by enchondral ossification. On the other hand, van den Berg *et al.*[18] reported the induction of osteophytes by intra-articular injection of transforming growth factor (TGF)-β1, and suggested the possible role of this cytokine in their development.

Apoptosis is an important cellular process involved in the control of normal and pathological enchondral ossification[7,8]. In this study, we first demonstrated that apoptosis of chondrocytes is involved in the formation of human chondro-osteophytes within the OA joint. The results of TUNEL stain showed that DNA strand breaks of chondrocytes were most abundantly seen in the hypertrophic zone. EM observations confirmed the degenerative and apoptotic changes including nuclear shrinkage and chromatin condensation of the cells in this zone, which was similar to the appearance of these cells in the growth plate as previously reported[6]. EM analysis also indicated that a proportion of chondrocytes in the proliferative and hypertrophic zone underwent apoptosis, whereas others swell further, degenerate or die. It is important to know if these degenerative chondrocytes are also positive for TUNEL stain or not. Although TUNEL stain detected approximately 30% of chondrocytes in the hypertrophic zone, only 3–5% of chondrocytes were morphologically apoptotic under EM. Additional study is under way to investigate this non-specificity of TUNEL stain using TUNEL–TEM method.

There is sufficient evidence to suggest that NO production can lead to apoptosis of chondrocytes and cartilage degeneration in OA joints[9–14]. Furthermore, NO influences collagen and proteoglycan synthesis by chondrocytes, interferes with chondrocyte migration and attachment to fibronectin, and promotes chondrocyte apoptosis[19]. Previous studies have also shown high concentrations of NO in the arthritic synovium, articular cartilage and synovial fluid, and that chondrocytes are probably the major cell source of NO[20,21]. It is known that inflammatory cytokines such as IL-1, IL-17, TNF or lipopolysaccharide (LPS) stimulate the expression of iNOS in chondrocytes, whereas TGF-β, IL-4, and IL-10 inhibit iNOS expression[9,12–15]. The expression of

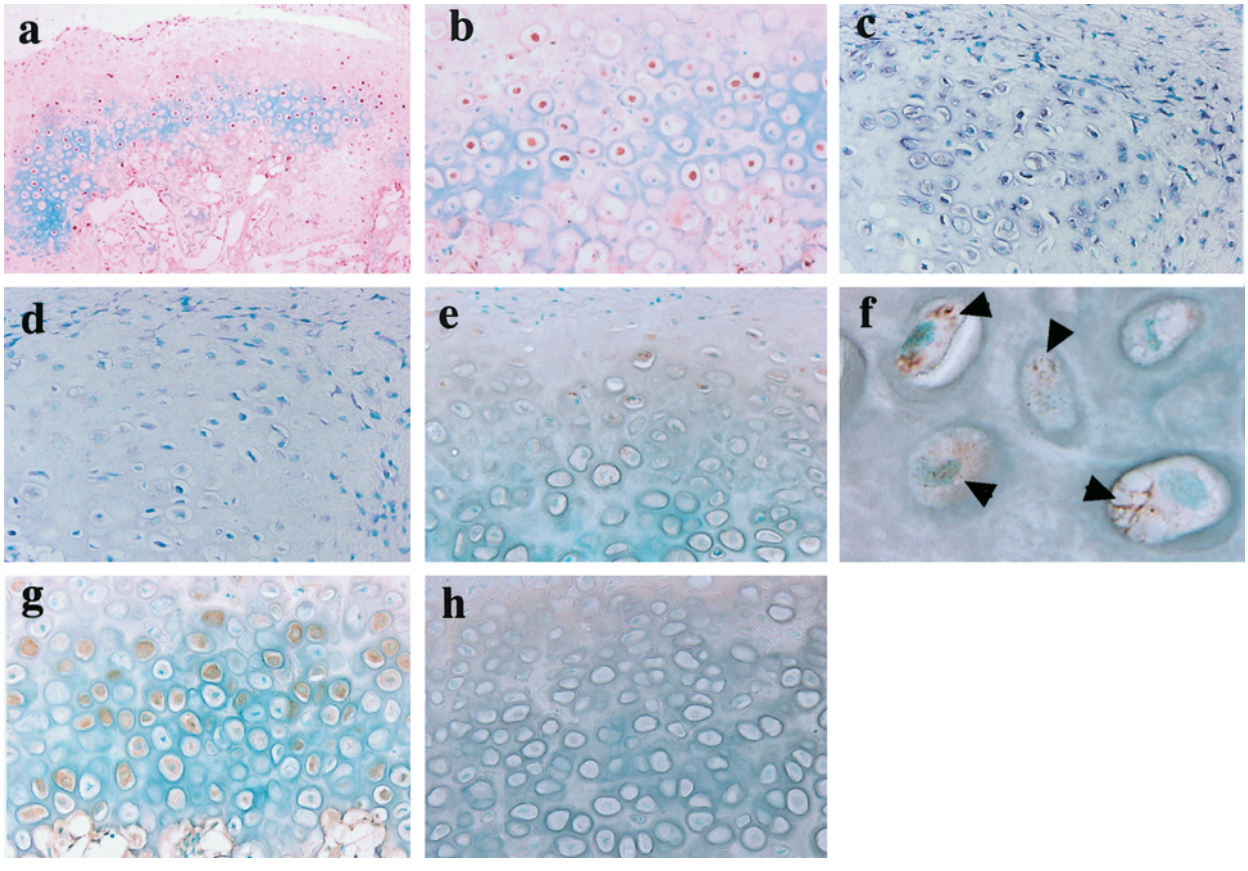

Fig. 2. (a) *In situ* detection of apoptosis by TUNEL stain in chondro-osteophyte. Approximately 30% of hypertrophic chondrocytes were TUNEL-positive. Original magnification: ×25. (b) High power magnification of Fig. (a). Original magnification: ×50. (c) *In situ* hybridization of inducible nitric oxide synthase (iNOS) mRNA (red-colored signals). Original magnification: ×50. (d) Negative control of Fig. (c) using sense probe for iNOS mRNA. (e) Immunostain for iNOS. Note the distribution of iNOS positive cells in proliferative and hypertrophic zone. Original magnification: ×50; (f) High power magnification of Fig. (e). Note the cytoplasmic or nuclear localization of iNOS protein. Oil immersion. (g) Immunostain for nitrotyrosine, a marker of nitric oxide-dependent oxidative damage, showing cytoplasmic localization at proliferative and hypertrophic chondrocytes. Original magnification: ×50. (h) Negative control for iNOS and nitrotyrosine immunostain. Primary antibodies were omitted. Original magnification: ×50.

iNOS on chondrocytes is partly regulated by a balance between these cytokines in the microenvironment[12]. Moreover, it is clear that chondrocytes at the periphery of OA joints are exposed to altered biomechanical forces. Das *et al*.[22] reported that NO release by chondrocytes increased in response to the duration and magnitude of the fluid-induced shear stress and that the high concentrations of NO in turn promoted cell death. Interestingly, iNOS-deficient mice with experimentally-induced OA show markedly reduced osteophyte formation and minimal OA lesions[23]. Pelletier *et al*.[24] showed in the treatment of OA dog model with N-iminoethyl-L-lysine, a selective inhibitor of iNOS, that the growth of osteophyes were reduced, in association with a decrease in IL-1 production. That osteophyte formation is a physiological reaction to abnormal mechanical forces acting on damaged articular surfaces of the unstable joints argues against a direct role for NO in the development of chondro-osteophytes. However, based on the present results, chondrocytes forming chondro-osteophytes can be affected by both biochemical and biomechanical factors to upregulate iNOS synthesis and catalyse NO formation.

Table I
Semi-quantitative anlaysis of DNA fragmentation and NO production in chondrocytes

	Chondro-osteophyte zones (n=12)							
	Proliferative zone				Hypertrophic zone			
	−	+	++	+++	−	+	++	+++
Tunnel	0	7	5	0	0	3	9	0
iNOS	0	3	4	5	0	1	3	8
Nitrotyrosine	0	0	6	6	0	1	6	5

Positive cell ratios labeled by TUNEL method or specific antibodies for inducible nitric synthase or nitrotyrosine were expressed; (−) no cells staining, (+) 0–25% of cells positive, (++) 26–50% of cells positive, (+++) >50% of cells positive.

NO reacts rapidly with superoxide anions and is easily converted to related cytotoxic molecules such as peroxynitrite or nitrite. The presence of peroxynitrite can be determined by the formation of protein nitrotyrosine[9]. In chondro-osteophytes, nitrotyrosine is also diffusely distributed over the cartilagenous area, which might cause chondrocyte DNA strand breaks, lipid and protein modification, and promote apoptosis. The exact mechanism of induction of chondrocyte apoptotic cell death following exposure to NO is still unknown. As shown in Table I, TUNEL positive ratio was relatively fixed (S.E.M.=5.7%

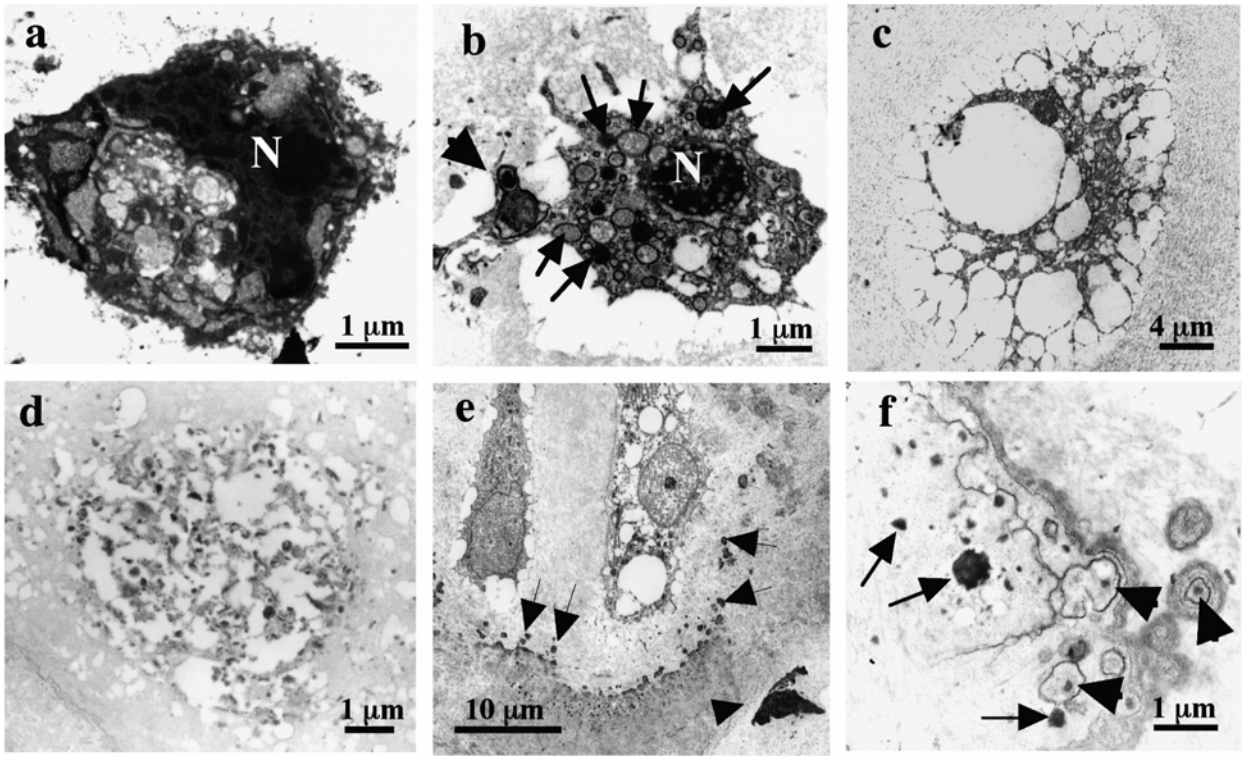

Fig. 3. Electron micrographs of chondrocytes showing various types and stages of cell death in the hypertrophic zone of chondro-osteophyte. (a) Typical appearance of chondrocytes undergoing apoptosis with chromatin condensation and margination in the nucleus (N). (b) Late stage of apoptotic cell death with condensed chromatin in the nucleus (N), vesicular bodies and electron-dense granules (arrowheads), which is giving off the apoptotic bodies into the matrix (arrow). (c) Hypertrophic chondrocyte with degenerative changes. Note the expansion of territorial matrix. (d) Cellular debris within pericellular matrix, presumably of hypertrophic chondrocyte origin. (e) Hypertrophic chondrocytes adjacent to the calcification area in the deep hypertrophic zone. Chondrocytes containing abundant cytoplasm with several organelles (left), chondrocytes showing paucity of cytoplasmic organelles (middle), and apoptotic chondrocyte consisting of electron dense materials (bottom right, arrow head) are seen. Note the calcification around each extracellular vesicle (small arrows). (f) Extracellular vesicles packed together and surrounded by calcifying matrices. They are morphologically heterogenious and contain cell debris (arrow) and matrix vesicles (arrow heads), suggesting chondrocyte fragments and matrix vesicles may act as a scaffold for the initial calcification.

and 4.5% in the proliferative and hypertrophic zones, respectively). These data might suggest the proper regulation of cell death by some control genes. Activated p53 tumor suppressor gene accumulates in nuclei after DNA damage, which in turn regulates cell cycle progression and apoptosis[25]. It is possible that peroxynitrite can induce accumulation of p53 protein and its related proteins, such as Bcl-2 or Bax in chondrocytes and regulate cell survival.

The role of chondrocyte apoptotic death in the development of chondro-osteophytes is of greatest interest. In the course of cell death, chondrocytes were surrounded by calcified matrix. Our results indicated that initial calcification occurred around each matrix vesicle derived by viable chondrocytes or cell debris. Extracellular vesicles containing new woven bone and cartilage bars of the primary trabeculae appear to be directly absorbed by osteoclasts followed by osteoblastic new bone formation (data not shown). Anderson[26] suggested that the formation of matrix vesicles in the growth plate by vesiculating apoptotic chondrocytes may reflect an additional useful effect of programmed cell death; the effect could be the generation of extracellular vesicles that are involved in the calcification process. Recently, Hashimoto *et al.*[27] reported the expression of functional apoptotic bodies derived from OA chondrocytes, which may contribute to the pathological calcification. Taken together, these studies and the present results suggest that chondrocytes in the hypertrophic zone of chondro-osteophytes might play a central role in the selection of the initial site of matrix mineralization through their ability to synthesize matrix vesicles or apoptotic bodies. NO is involved in the chondro-osteophyte formation, and seemed to play a significant role in chondrocyte hypertrophy and cell death.

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